

# Direct Nitrous Oxide Emission from the Aquacultured Pacific White Shrimp (*Litopenaeus vannamei*)

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## ABSTRACT

The Pacific white shrimp (*Litopenaeus vannamei*) is widely used in aquaculture, where it is reared at high stocking densities, temperatures, and nutrient concentrations. Here we report that adult *L. vannamei* shrimp emit the greenhouse gas nitrous oxide (N<sub>2</sub>O) at an average rate of 4.3 nmol N<sub>2</sub>O/individual × h, which is 1 to 2 orders of magnitude higher than previously measured N<sub>2</sub>O emission rates for free-living aquatic invertebrates. Dissection, incubation, and inhibitor experiments with specimens from a shrimp farm in Germany indicated that N<sub>2</sub>O is mainly produced in the animal's gut by microbial denitrification. Microsensor measurements demonstrated that the gut interior is anoxic and nearly neutral and thus is favorable for denitrification by ingested bacteria. Dinitrogen (N<sub>2</sub>) and N<sub>2</sub>O accounted for 64% and 36%, respectively, of the nitrogen gas flux from the gut, suggesting that the gut passage is too fast for complete denitrification to be fully established. Indeed, shifting the rearing water bacterial community, a diet component of shrimp, from oxic to anoxic conditions induced N<sub>2</sub>O accumulation that outlasted the gut passage time. Shrimp-associated N<sub>2</sub>O production was estimated to account for 6.5% of total N<sub>2</sub>O production in the shrimp farm studied here and to contribute to the very high N<sub>2</sub>O supersaturation measured in the rearing tanks (2,099%). Microbial N<sub>2</sub>O production directly associated with aquacultured animals should be implemented into life cycle assessments of seafood production.

## IMPORTANCE

The most widely used shrimp species in global aquaculture, *Litopenaeus vannamei*, is shown to emit the potent greenhouse gas nitrous oxide (N<sub>2</sub>O) at a particularly high rate. Detailed experiments reveal that N<sub>2</sub>O is produced in the oxygen-depleted gut of the animal by bacteria that are part of the shrimp diet. Upon ingestion, these bacteria experience a shift from oxic to anoxic conditions and therefore switch their metabolism to the anaerobic denitrification process, which produces N<sub>2</sub>O as an intermediate and dinitrogen (N<sub>2</sub>) gas as an end product. The N<sub>2</sub>O/N<sub>2</sub> production ratio is unusually high in the shrimp gut, because denitrification cannot be fully established during the short gut passage time of food-associated bacteria. Nitrous oxide emission directly mediated by *L. vannamei* contributes significantly to the overall N<sub>2</sub>O emission from aquaculture facilities.

Aquaculture facilities are characterized by large loads of nutrients, especially nitrogen compounds originating from the protein-rich feed. Accordingly, they are sites of intense nitrogen turnover, including the microbial processes of nitrification and denitrification (1, 2). Both processes produce nitrous oxide (N<sub>2</sub>O), which is a potent greenhouse gas and ozone-depleting substance (3, 4). Aquaculture was recently discussed as an important source of atmospheric N<sub>2</sub>O (2, 5). It was estimated that total N<sub>2</sub>O emissions from aquaculture currently account for 0.09 to 0.12 Tg N/year and will rise to 0.38 to 1.01 Tg N/year by 2030 due to the rapidly growing aquaculture industry (2, 5, 6). The maximum projection of N<sub>2</sub>O emissions from aquaculture for 2030 would correspond to 5.7% of the current estimate of global N<sub>2</sub>O emissions (2). Unfortunately, direct measurements of N<sub>2</sub>O emissions from aquaculture are missing, and the current estimates are based on the overall nitrogen load of aquaculture facilities and N<sub>2</sub>O emission factors that were derived from wastewater treatment plants (2, 5, 7). It is highly uncertain whether these emission factors reflect the true N<sub>2</sub>O yield of nitrogen cycling processes in aquaculture facilities, since little is known about the mechanisms and controlling factors of microbial N<sub>2</sub>O production in aquaculture facilities (7).

Established compartments for microbial nitrogen turnover in aquaculture facilities are the water column, suspended organic

particles, and the biofilm-covered walls of the rearing tanks. In recirculating aquaculture systems (RASs), the rearing water is biologically treated in filter units to prevent accumulation of toxic nitrogen compounds, such as ammonia and nitrite, in the rearing tanks. Just as in wastewater treatment plants, one of the main purposes of these biofilters is to eliminate inorganic nitrogen compounds through microbial conversion to dinitrogen (N<sub>2</sub>) through denitrification and/or anammox (8). However, the production and emission of N<sub>2</sub>O are often undesired side effects during the microbial processing of wastewater (9).

In this study, an additional compartment of microbial N<sub>2</sub>O production in aquaculture facilities, namely, the guts of the reared animals, is proposed and investigated. In many free-living terrestrial, freshwater, and marine invertebrates, the gut is a distinct

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TABLE 1 *In situ* conditions in rearing tank

Variable	Mean $\pm$ SD
Temperature ( $^{\circ}$ C)	29.5 $\pm$ 0.5
Salinity <sup>a</sup>	19 $\pm$ 2
pH	8.08 $\pm$ 0.14
NO <sub>3</sub> <sup>-</sup> concentration (mmol/liter)	9.13 $\pm$ 3.73
NH <sub>4</sub> <sup>+</sup> concentration (mmol/liter)	0.024 $\pm$ 0.007
NO <sub>2</sub> <sup>-</sup> concentration (mmol/liter)	0.008 $\pm$ 0.003
N <sub>2</sub> O concentration (nmol/liter)	140 $\pm$ 59
N <sub>2</sub> O atmospheric saturation (%)	2,099 $\pm$ 877
Adult <i>L. vannamei</i> stocking density	
Shrimp/m <sup>2</sup>	100
Shrimp/liter	0.5

<sup>a</sup> Salinity is a dimensionless variable (65).

compartment of microbial nitrogen turnover and, in particular, a site of significant N<sub>2</sub>O production (10–12). The guts of earthworms, freshwater insect larvae, and marine copepods are characterized by the absence of oxygen and/or the presence of nitrate (NO<sub>3</sub><sup>-</sup>) and labile organic carbon, which together stimulate the denitrification activity of ingested bacteria (13–15). During the feeding processes of these invertebrates, facultative denitrifying bacteria from the ambient oxic environment are abruptly transferred into the anoxic gut (11, 16). Such oxic-anoxic shifts cause transiently high N<sub>2</sub>O yields of the denitrification process (i.e., a high ratio of N<sub>2</sub>O production to N<sub>2</sub>O plus N<sub>2</sub> production), due to delayed induction of the N<sub>2</sub>O reductase (17, 18).

The most important invertebrate used in aquaculture worldwide is the Pacific white shrimp *Litopenaeus vannamei* (Crustacea, Penaeidae). In 2008, this species made up 2.3 of the 5.0 million tons of crustaceans globally produced in aquaculture (19). *L. vannamei* is often reared in superintensive aquaculture systems with high stocking densities, temperatures, and nutrient concentrations (19–21). It follows that shrimp guts are potential compartments of N<sub>2</sub>O production at very high abundance in the rearing tanks (up to 100 adult shrimp/m<sup>2</sup> [21, 22]). Therefore, we investigated the rate and mechanism of N<sub>2</sub>O production directly associated with *L. vannamei* and its gut. The aims were (i) to measure the overall N<sub>2</sub>O emission rate of *L. vannamei* under nearly *in situ* conditions and the N<sub>2</sub>O saturation level in the rearing water, (ii) to establish the shrimp gut as a compartment of N<sub>2</sub>O production in aquaculture systems, by combined dissection, incubation, and inhibitor experiments, (iii) to characterize the gut microenvironment (O<sub>2</sub> and pH) with microsensor measurements, and (iv) to test the hypothesis that a sudden shift from oxic to anoxic conditions induces unbalanced denitrification in the bacterial community in the rearing water and thus high N<sub>2</sub>O yields of gut denitrification.

## MATERIALS AND METHODS

**Nitrous oxide emission from complete animals and dissected guts.** *L. vannamei* shrimp were obtained from an indoor RAS in Affinghausen (Germany), designed and supervised by Polyplan GmbH (Bremen, Germany). Rearing conditions are summarized in Table 1. Shrimp, with a wet weight of 20.4  $\pm$  7.3 g (mean  $\pm$  standard deviation [ $n = 36$ ]), were kept in original rearing water until used for experiments. The animals were killed in ice water immediately prior to incubation experiments or gut dissection. Killing was necessary because living animals immediately evacuated their guts when transferred to incubation bottles (probably as a stress response), which would have compromised the study of gut denitrifica-

tion. To avoid artifacts due to incipient decay processes, incubations of freshly killed shrimp were generally limited to 2 h, during which the production of N<sub>2</sub>O was linear. Complete animals were incubated in 100-ml glass bottles that contained 30 ml of aerated, 0.2- $\mu$ m-filtered, rearing water and an air-filled headspace. The bottles were sealed gastight with butyl rubber stoppers.

For incubation of intact guts, freshly killed animals were dissected along their dorsal side with scissors and the guts were carefully removed with sterile forceps. Dissected complete guts (i.e., gut contents and gut wall but excluding digestive glands) were incubated in 6-ml Exetainer vials (Labco, High Wycombe, United Kingdom) that contained 1 ml of aerated, 0.2- $\mu$ m-filtered, rearing water and an air-filled headspace.

In addition to these oxic incubations, dissected complete guts and gut walls were incubated under anoxic conditions in 6-ml Exetainer vials that contained 1 ml of N<sub>2</sub>-flushed, 0.2- $\mu$ m-filtered, rearing water. After the vials were closed for anoxic incubation, the headspace was flushed again with N<sub>2</sub> for 5 to 10 min. As a negative control, N<sub>2</sub>-flushed, 0.2- $\mu$ m-filtered, rearing water was incubated in gastight 100-ml glass bottles; the minute rate of the negative control was subtracted from the rate obtained for the gut samples.

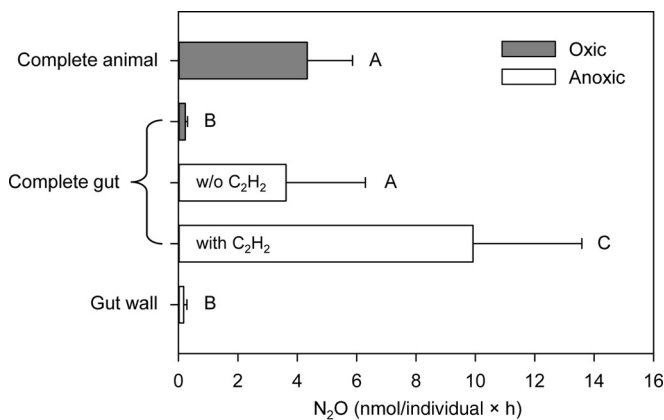
For each incubation assay, 4 to 15 replicates were run with 1 shrimp or 1 dissected gut per incubation vial. Incubations were conducted at a mean temperature of 28  $\pm$  2 $^{\circ}$ C for up to 3 h. The incubation vials were placed on a shaker to enforce the equilibration of N<sub>2</sub>O between the water and the headspace. N<sub>2</sub>O accumulation was monitored by regularly taking 1-ml headspace samples through the rubber stopper (every 15 to 60 min). The analysis of N<sub>2</sub>O production by gas chromatography (GC 7890; Agilent Technologies) and the calculation of N<sub>2</sub>O emission rates were as described previously (12).

The potential rate of total denitrification (i.e., the rate of production of N<sub>2</sub>O plus N<sub>2</sub>) of dissected complete guts was measured with the acetylene inhibition technique (23). Freshly dissected guts were incubated in 6-ml Exetainers with 1 ml of N<sub>2</sub>-flushed, 0.2- $\mu$ m-filtered, rearing water and a headspace containing a 1:10 mixture of acetylene and N<sub>2</sub>. Sampling, N<sub>2</sub>O analysis, and calculation of N<sub>2</sub>O production rates were performed as described above.

The *in situ* concentration of N<sub>2</sub>O in the rearing water was determined by adding 100 ml unfiltered rearing water to 125-ml gastight bottles ( $n = 10$ ) that contained 4 ml saturated HgCl<sub>2</sub> to stop any biological activity. The bottles were shaken for several hours for equilibration of N<sub>2</sub>O between the water and the headspace, and the N<sub>2</sub>O concentration in the headspace was measured as described above. The N<sub>2</sub>O concentration in the water was calculated as described previously (24).

**Gut microenvironment.** Microsensors for O<sub>2</sub> and pH measurements in dissected guts were constructed as described previously (25, 26). The tip diameters of the sensors were 10 to 20  $\mu$ m. The sensors were calibrated before, during, and after the measurements. Oxygen microsensors were calibrated in Ringer's solution (Merck, Germany) at 0 and 100% air saturation by N<sub>2</sub> and air flushing, respectively. The pH sensors were calibrated in standard solutions of pH 7 and pH 9. Freshly dissected guts were fixed on an agarose bottom in a flow cell, which was continuously flushed with aerated Ringer's solution (14). Aided by a dissection microscope, the microsensor tip was positioned at the outer surface of the gut wall, which was then defined as depth zero. Radial concentration profiles through the gut were started 1 mm above the upper gut wall in the aerated Ringer's solution and continued in increments of 0.1 mm to 3 mm below the lower gut wall, into the agarose bottom. Flattening of the gut due to insertion of the thin-tipped microsensors was negligible. Profiles were determined in the foregut, midgut, and hindgut at different degrees of gut filling. All measurements and calibrations were performed at 28 $^{\circ}$ C.

**Oxic-anoxic shift imposed on bacteria in rearing water.** Unfiltered rearing water containing free-living and particle-attached bacteria was aerated and added to 100-ml glass bottles ( $n = 3$ ) that were sealed with butyl rubber stoppers. The water was continuously stirred with a glass-coated magnetic stirring bar. Water samples (3 ml) were taken every 20



**FIG 1** Rates of N<sub>2</sub>O emission from the shrimp *Litopenaeus vannamei* and its dissected guts incubated in filtered rearing water at 28°C. Complete animals were incubated under oxic conditions, dissected complete guts (i.e., gut contents plus gut wall) were incubated under oxic and anoxic conditions, and gut walls were incubated under anoxic conditions. Complete guts were also incubated under anoxic conditions with 10% acetylene (C<sub>2</sub>H<sub>2</sub>), which inhibits the last step of denitrification; the resulting N<sub>2</sub>O production indicates total denitrification. Means ± standard deviations are shown ( $n = 4$  to 15). Treatments that share the same letter are not significantly different (see the text for details on statistics).

min through the stopper and transferred to N<sub>2</sub>-flushed 6-ml Exetainers. After 1 h of oxic incubation, the water in the bottles was flushed with N<sub>2</sub> for 10 min, the bottles were sealed again, and the remaining headspace was flushed with N<sub>2</sub> for 5 min. After this oxic-anoxic shift, water samples (3 ml) were taken at intervals for a total incubation period of 16 h. The N<sub>2</sub>O concentration in the headspace was measured after forced equilibration of N<sub>2</sub>O between the water and the gas phase. Calculation of N<sub>2</sub>O production rates was performed as described above.

## RESULTS AND DISCUSSION

**Nitrous oxide emission from complete animals and dissected guts.** Under simulated *in situ* conditions of the rearing tanks (Table 1), freshly killed aquacultured shrimp (*L. vannamei*) emitted N<sub>2</sub>O with a rate of  $4.3 \pm 1.5$  nmol/individual × h (mean ± standard deviation [ $n = 15$ ]), which is equivalent to a biomass-specific rate of  $0.20 \pm 0.07$  nmol/g (wet weight) × h (Fig. 1). On an individual basis, this is the highest N<sub>2</sub>O emission rate recorded to date for any aquatic invertebrate species. In fact, this rate is 14 to 62 times higher than the mean rates of free-living freshwater (0.07 nmol/individual × h) or marine (0.32 nmol/individual × h) invertebrates (11, 12). Conversely, the biomass-specific N<sub>2</sub>O emission rate of *L. vannamei* is 3 to 4 times lower than the mean biomass-specific rates of free-living freshwater (0.85 nmol/g [wet weight] × h) or marine (0.60 nmol/g [wet weight] × h) invertebrates (11, 12).

The N<sub>2</sub>O emitted from *L. vannamei* was mainly produced inside the animal's gut (Fig. 1). Dissected complete guts (i.e., gut contents plus gut wall) incubated under anoxic conditions produced N<sub>2</sub>O at  $3.6 \pm 2.7$  nmol/gut × h (mean ± standard deviation [ $n = 13$ ]), which was not significantly different from the N<sub>2</sub>O emission rate for the complete animal ( $P = 0.666$ , one-way analysis of variance [ANOVA] followed by the Holm-Šidák *post hoc* test). In contrast, dissected gut walls produced significantly less N<sub>2</sub>O than complete guts under anoxic conditions ( $P < 0.001$ ). Hence, N<sub>2</sub>O production mainly took place in the gut contents rather than in the gut wall, which strongly suggests that N<sub>2</sub>O pro-

duction is mediated by ingested microbes rather than by microbes colonizing the gut wall or by specific symbionts. Complete guts incubated under oxic conditions produced significantly less N<sub>2</sub>O than complete guts incubated under anoxic conditions ( $P = 0.020$ ). This observation indicates that, in the shrimp gut, N<sub>2</sub>O production is due to anaerobic denitrification rather than aerobic nitrification. To test for denitrification, acetylene (C<sub>2</sub>H<sub>2</sub>) was added to complete guts incubated anoxically, to inhibit the last reduction step of denitrification (23). Nitrous oxide was then produced at a rate of  $9.9 \pm 3.7$  nmol/gut × h (mean ± standard deviation [ $n = 9$ ]), which was significantly higher than the N<sub>2</sub>O production rate for complete guts incubated anoxically but without C<sub>2</sub>H<sub>2</sub> ( $P < 0.001$ ). The N<sub>2</sub>O production rate in the presence of C<sub>2</sub>H<sub>2</sub> is interpreted as the total denitrification rate in the shrimp gut. For comparison, total denitrification rates measured in dissected guts of freshwater invertebrates and earthworms are approximately 0.5 and 1.2 to 6.6 nmol/gut × h, respectively (11, 27). Total denitrification in the shrimp gut produced 64% N<sub>2</sub> and 36% N<sub>2</sub>O, as calculated from N<sub>2</sub>O production rates measured in the presence and absence of C<sub>2</sub>H<sub>2</sub>.

**Gut microenvironment.** Microsensor measurements through dissected *L. vannamei* guts (still filled with food particles) revealed anoxic conditions through almost the entire gut diameter (Fig. 2A). Thus, O<sub>2</sub> diffusing from the air-saturated Ringer's solution into the gut was efficiently consumed inside the gut. Even empty guts were not fully oxygenated, which suggests that the gut walls, the epithelium itself, and/or some residential gut bacteria contribute to O<sub>2</sub> consumption (Fig. 2B). No obvious variation in the radial O<sub>2</sub> profiles along the length axis of filled or empty guts was observed (Fig. 2A and B). It should be kept in mind, however, that the transport of food particles is interrupted due to dissection, which may obscure axial concentration gradients. Under *in vivo* conditions, the O<sub>2</sub> flux into the gut is probably much lower than after dissection. The hemolymph surrounding the gut typically has a much lower O<sub>2</sub> concentration (approximately 2 to 3 μmol/liter) than the air-saturated Ringer's solution (28, 29). Therefore, it can be safely assumed that, under *in vivo* conditions, the entire gut is anoxic even when not completely filled. The largely anoxic conditions in the shrimp gut seem to be common for invertebrates, since previous microsensor measurements in the guts of diverse species all showed similar results (13–15, 30, 31).

The pH in the gut lumen was 7.6 to 7.8 and thus slightly lower than that of the rearing water (pH 8.1) and higher than that of the Ringer's solution (pH 7.0 to 7.1) (Fig. 2C). No significant variation of the radial pH profiles along the length axis of the gut was observed (data not shown). The gut pH in *L. vannamei* is thus similar to that of other marine detritivores (30). Extreme values that might enable the digestion of refractory organic matter or create exclusive environments for gut symbionts, as in many terrestrial invertebrates, were not observed (32, 33). Gut pH might also be influenced by the metabolism of ingested or residential microorganisms (30). In fact, microbial denitrification activity increases the pH by alkalinity production, which may explain the higher gut pH relative to the Ringer's solution.

The O<sub>2</sub> and pH microenvironment of the *L. vannamei* gut is favorable for microbial denitrification. Complete anoxia and nearly neutral pH values in the center of the gut should favor complete denitrification to N<sub>2</sub> (which accounted for 64% of the nitrogen gas flux), rather than incomplete denitrification to N<sub>2</sub>O (which accounted for 36% of the nitrogen gas flux). The activity of



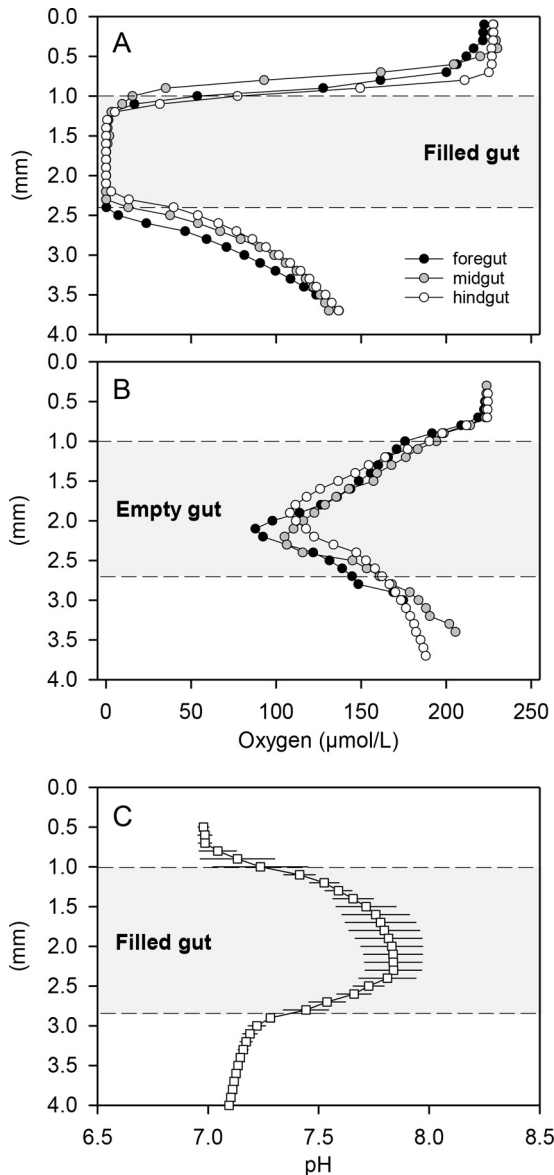


FIG 2 Radial microprofiles of  $O_2$  and pH through dissected guts of *L. vannamei*. Guts were fixed on agarose in a flow cell that was continuously supplied with air-saturated Ringer's solution. Gray area, gut interior; dashed lines, gut walls; upper white area, Ringer's solution; lower white area, agarose. (A) Oxygen concentration profiles through filled foreguts, midguts, and hindguts. (B) Oxygen concentration profiles through empty foreguts, midguts, and hindguts. In panels A and B, single representative profiles are shown. (C) pH profile (mean  $\pm$  standard deviation [ $n = 6$ ]) through filled guts, calculated from pH profiles measured in foreguts, midguts, and hindguts.

the  $N_2O$  reductase is partially inhibited by low pH values (34, 35), which were not observed in the *L. vannamei* gut. Therefore, the best explanation for the large fraction of  $N_2O$  in the total nitrogen flux from the gut is that the gut passage time is too short to allow full establishment of complete denitrification to  $N_2$ . Partial inhibition of  $N_2O$  reductase by low  $O_2$  concentrations (36) might occur close to the gut wall, however, where  $O_2$  is present in trace amounts (Fig. 2A).

Due to the largely anoxic conditions inside the *L. vannamei* gut, it is unlikely that nitrification contributes to  $N_2O$  production

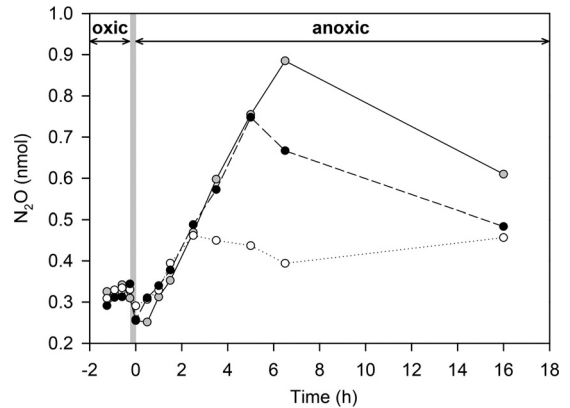


FIG 3 Induction of  $N_2O$  production in unfiltered rearing water by an oxic-anoxic shift. White areas with arrows, oxic and anoxic periods of the incubation experiment; gray area, transition period. Results from three replicate incubations are shown.

directly in the gut, where the bulk of  $N_2O$  emitted by *L. vannamei* has its origin. The microoxic conditions close to the gut wall, however, may allow nitrifier denitrification, a process known to produce  $N_2O$  in the near absence of  $O_2$  (37). Nitrification might contribute to the total  $N_2O$  emission by *L. vannamei* if oxygenated biofilms are present on the body surface of the animal, as was observed for other marine and freshwater invertebrate species (12, 38–40). For *L. vannamei*, however, exoskeletal  $N_2O$  production must be very low or even absent, because the anoxic gut alone emits  $N_2O$  at the same rate as the complete animal (Fig. 1).

**Oxic-anoxic shift imposed on bacteria in rearing water.** When the bacterial community of the rearing water was experimentally transferred from oxic to anoxic conditions,  $N_2O$  production immediately increased from  $0.20 \pm 0.28$  nmol/liter  $\times$  h to  $0.92 \pm 0.18$  nmol/liter  $\times$  h (Fig. 3). This indicates that the oxic-anoxic shift prompted facultative anaerobic bacteria to switch rapidly from aerobic respiration to denitrification, with initially unbalanced enzyme activities. The accumulation of  $N_2O$  in the incubation vials continued for 2.5 to 6.5 h, after which the  $N_2O$  concentration started to decrease (Fig. 3). The gut passage time in *L. vannamei* is approximately 1 h (41, 42); therefore, the food bolus would have moved through the first quarter of the gut (i.e., the foregut) during the time it took to complete the oxic-anoxic shift of the rearing water (i.e., 15 min). The microsensor profiles revealed anoxic conditions in the foregut, and thus the experimentally induced oxic-anoxic shift was not unrealistically fast. Notably, the total gut passage time is considerably shorter than the time required for the bacterial inoculum from the rearing water to reach the phase of net  $N_2O$  consumption. Thus, it is likely that many bacteria in the gut contents of *L. vannamei* have low  $N_2O$  reductase activities, which is consistent with a high  $N_2O$  yield for gut denitrification. The  $N_2O$  yield of 36% for gut denitrification in *L. vannamei* is in the same range as observed for gut denitrification in earthworms and freshwater insect larvae (11, 43). Similar  $N_2O$  yields were also measured when complete animals were incubated, which argues against a dissection artifact (11, 44). Nitrous oxide yields for gut denitrification are thus much higher than the <1% typically measured in the water column and sediments of aquatic ecosystems under undisturbed conditions (45, 46).

Transient  $N_2O$  accumulation after shifts from oxic to anoxic

conditions is also known from pure cultures of denitrifiers (17, 18, 47, 48). The time required to balance enzyme activities and then perform complete denitrification after the oxic-anoxic shift varies with the species and culture conditions. Thus, the community composition of denitrifiers ingested by *L. vannamei* might influence the temporal patterns of N<sub>2</sub>O production and consumption and eventually the N<sub>2</sub>O yield of gut denitrification. Furthermore, knowing the community composition of gut denitrifiers could be revealing, because N<sub>2</sub>O-respiring taxa constitute only 10 to 15% of all known denitrifying taxa (49). In the highly NO<sub>3</sub><sup>-</sup>-enriched RAS, N<sub>2</sub>O-respiring taxa may in fact be underrepresented, since N<sub>2</sub>O reduction is inhibited by high NO<sub>3</sub><sup>-</sup> (50, 51).

In summary, N<sub>2</sub>O production associated with *L. vannamei* is likely due to denitrification by ingested bacteria in the anoxic gut of the animal. This interpretation is in accordance with observations on terrestrial and aquatic invertebrates, for which ingested denitrifiers are the key players in N<sub>2</sub>O production (11, 52, 53). The bacterial abundance in aquaculture water is generally high, due to the copious supplies of inorganic and organic nutrients, and can reach up to  $3.9 \times 10^8$  cells/ml in RASs (21, 54, 55). *L. vannamei* mainly takes up particle-attached microorganisms by feeding on suspended particulate organic matter, and it uses these microorganisms as an additional food source (56, 57). This study uncovered an additional role of microorganisms ingested by *L. vannamei*, i.e., the production of N<sub>2</sub>O through denitrification by microorganisms that survive and remain or become metabolically active during the gut passage.

Nitrous oxide produced in the *L. vannamei* gut is first released into the rearing water and then expelled into the atmosphere, due to the intense aeration of the rearing tanks. Nevertheless, the steady-state N<sub>2</sub>O concentration in the rearing water of the shrimp farm studied here corresponded to 2,099% atmospheric saturation (Table 1). The oxic conditions in the rearing tanks exclude the possibility that this N<sub>2</sub>O is converted by free-living bacteria capable of anaerobic denitrification. Similarly, N<sub>2</sub>O cannot be metabolized by aerobic nitrifying bacteria and archaea, because it is only a by-product and not a true intermediate of ammonia oxidation. To date, assimilation of N<sub>2</sub>O into the microbial biomass has been reported only for N<sub>2</sub>-fixing cyanobacteria (58), which are not abundant in the nitrogen-rich rearing water of shrimp farms. Thus, the most likely fate of N<sub>2</sub>O produced by *L. vannamei* is emission into the atmosphere.

**Significance of direct N<sub>2</sub>O emission from *L. vannamei*.** In light of the fast-growing aquaculture industry, especially for penaeid shrimp species such as *L. vannamei*, direct N<sub>2</sub>O emissions from reared animals need to be constrained and integrated into whole-aquaculture emission budgets (2). For the RAS from which the tested shrimp were obtained, the relative contribution of N<sub>2</sub>O production directly associated with shrimp ( $R_{N_2O\text{-shrimp}}$ ) to the total N<sub>2</sub>O production in the rearing tank ( $R_{N_2O\text{-tank}}$ ) was estimated. Assumptions for this estimate were that the gas flux between the rearing water and the air was at steady state and that the rearing tank formed one well-mixed unit with the biofilter operating in recirculation. According to reference 59,  $R_{N_2O\text{-tank}}$  can be described as follows:  $R_{N_2O\text{-tank}} = V_{\text{tank}} \times k_{N_2O} \times (C_{N_2O\text{-tank}} - C_{N_2O\text{-sat}})$ , where  $V_{\text{tank}}$  is the volume of the rearing tank (53 m<sup>3</sup>),  $k_{N_2O}$  is the volumetric mass transfer coefficient for N<sub>2</sub>O (see below),  $C_{N_2O\text{-tank}}$  is the N<sub>2</sub>O concentration measured in the rearing tank ( $140 \pm 59$  nmol/liter) (Table 1), and  $C_{N_2O\text{-sat}}$  is the N<sub>2</sub>O equilibrium concentration at the given temperature and salinity

(6.7 nmol/liter). The parameter  $k_{N_2O}$  was estimated as follows. Based on the aeration rate (40 m<sup>3</sup>/h) and the bottom area (265 m<sup>2</sup>) of the rearing tank, the superficial gas velocity ( $v_{\text{GAS}}$ ) was calculated to be  $0.000042 \text{ m}^3/\text{m}^2 \times \text{s}$ . This value was used in an empirical equation derived in reference 59 to calculate  $k_{N_2O}$  as  $34,525 \times v_{\text{GAS}}^{0.86}$ , i.e.,  $5.9 \text{ day}^{-1}$ .  $R_{N_2O\text{-tank}}$  was thus estimated to be  $42.0 \pm 17.5$  mmol/day (mean  $\pm$  standard deviation [ $n = 10$  replicate measurements of  $C_{N_2O\text{-tank}}$ ]).  $R_{N_2O\text{-shrimp}}$  amounted to  $4.3 \text{ nmol}/\text{individual} \times \text{h} \times 100 \text{ individuals}/\text{m}^2 \times 265 \text{ m}^2$ , i.e.,  $2.75 \pm 0.95$  mmol/day ( $n = 15$  replicate measurements of the individual-specific N<sub>2</sub>O emission rate). Thus,  $R_{N_2O\text{-shrimp}}$  was estimated to be  $6.5 \pm 3.5\%$  of  $R_{N_2O\text{-tank}}$ . This value may be different in other RASs with superintensive shrimp production but, to our knowledge, has not been quantified in any other shrimp farm.

The findings of this study should be implemented into life cycle assessments of shrimp production, which currently lack the aspect of N<sub>2</sub>O emissions (60). Mitigation strategies for animal-associated N<sub>2</sub>O emissions should aim at reducing the extremely high NO<sub>3</sub><sup>-</sup> concentrations in the rearing tanks, as gut denitrification is significantly reduced with depletion of ambient NO<sub>3</sub><sup>-</sup>, as shown for free-living aquatic invertebrates (61). Shrimp production accounts for only 6.35% of global aquaculture production (2), and thus the questions of whether other aquacultured animal species emit N<sub>2</sub>O directly and how much must be raised. The guts of fish and mollusks are potentially anoxic compartments in aquaculture systems in which anaerobic microbial processes such as denitrification might occur (62). For these animals, however, the availability of NO<sub>3</sub><sup>-</sup> inside the gut and the fate of gut-produced N<sub>2</sub>O are currently not known. For mollusks, including *Mytilus edulis*, significant N<sub>2</sub>O production also proceeds in microbial biofilms growing on the shells of the animals (40). This phenomenon can also be expected for the richly sculptured shells of oysters, which have been recognized as keystone species for coastal nitrogen management (63), but to date has not been noted for N<sub>2</sub>O emission, which may be a disadvantage of nitrogen removal stimulated by benthic macrofauna (39, 64). The current report on N<sub>2</sub>O emission from *L. vannamei* should thus inspire further research on N<sub>2</sub>O production directly associated with a larger variety of aquacultured and free-living animals.

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